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# The Cytokinesis-Block Micronucleus Technique and Its Application to Genotoxicity Studies in Human Populations

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**The development of the cytokinesis-block (CB) technique has made the human lymphocyte micronucleus assay (MN) a reliable and precise method for assessing chromosome damage. Recent studies in our laboratory have confirmed that this method is a sensitive indicator of *in vivo* radiation exposure in patients undergoing fractionated partial-body radiotherapy and rodents exposed to uniform whole-body irradiation, thus supporting the application of the cytokinesis-block micronucleus (CBMN) assay for biological dosimetry. To further define the use of this assay in biomonitoring, we have also undertaken extensive studies to determine the spontaneous level of MN in normal human populations and its relationship to various lifestyle factors. During the past year, we have also developed a new variation to the CBMN assay that enables the conversion of excision-repairable lesions to MN within one cell-cycle using cytosine arabinoside. With this method the slope of the *in vitro* dose–response curves was increased by a factor of 1.8 for X-rays, 10.3 for ultraviolet (254 nm) radiation, and approximately 40-fold for methylnitrosourea. Consequently, the CBMN assay can now be used not only to measure whole chromosome loss or chromosome breaks but also excision repair events. The versatility and simplicity of the CBMN assay together with new developments in automation should enable its successful application in monitoring exposed populations as well as identifying mutagen-sensitive individuals within a population.**

## Introduction

The level of genetic integrity of human populations is increasingly under threat due to industrial activities that result in exposure to chemical and physical genotoxins. Other factors that can influence genetic damage include lifestyle factors (e.g., diet), various medical therapies, and climatic changes (e.g., increased exposure to ultravoilet radiation due to depletion of atmospheric ozone). It is therefore important to be able to *a*) determine what is an acceptable level of genetic damage in a human population, *b*) identify individuals who could be hypersensitive to selected genotoxins, *c*) effectively screen new chemicals that are released into the environment, *d*) determine the level of increase in genetic damage in a population following a major accident, and *e*) routinely monitor individuals who are occupationally exposed to agents that can be detrimental at the genetic level.

A number of methodologies have been developed over the past 20 years, varying from cytogenetic techniques that can give a broad assessment of mutagenic events to adduct assays that are designed to detect exposure to specific agents. No one technique can satisfy all the requirements identified above, and it is now realized that a variety of methods can be combined to provide an effective screening system (*1,2*).

As part of our effort in this area of research, we have been actively involved in the development of micronucleus (MN) assays in human lymphocytes to measure both whole chromosome loss and chromosome breaks (Fig. 1). The MN methodology is simple and allows rapid assessment of cells, thus making it an economical procedure to

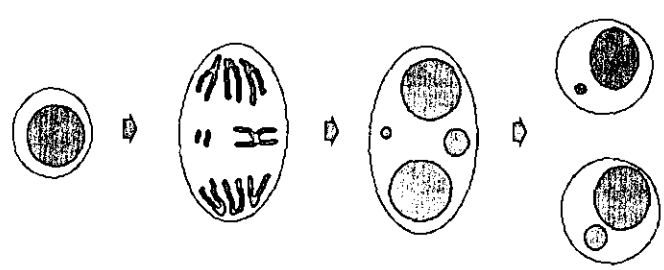

FIGURE 1. Micronucleus in a dividing nucleated cell. Micronuclei originate from either lagging chromosomes or chromosome fragments at anaphase. Micronuclei are readily identified because they are morphologically identical to but smaller than the main nuclei. A nondividing cell is unable to express its chromosome damage as micronuclei. Cells that have completed one nuclear division can be accumulated and identified as binucleate cells by adding cytochalsin-B, a cytokinesis blocking agent (*3,4*). Micronuclei are then scored in binucleated cells only.

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implement on a large scale. MN assays can only be effective as quantitative biological dosimeters if one can identify those cells that have divided after exposure because only dividing cells can express micronuclei. This goal was achieved by the development of the cytokinesis-block micronucleus (CBMN) technique, which uses cytochalasin-B to stop dividing cells from performing cytokinesis, thus allowing cells that have completed one nuclear division to be recognized by their binucleate appearance (*3,4*). As a consequence, the CBMN assay has been shown to be more accurate and more sensitive than the conventional methods that do not distinguish between dividing and nondividing cells (*4,5*). The development of the CBMN assay has enabled direct and valid comparisons with chromosome aberration assays. It has now been shown conclusively that the CBMN assay can detect between 60 and 90% of acentric fragments (*6,7*), although the relative efficiency specific for chromatid deletions as opposed to chromosome deletions has yet to be defined. Furthermore, the CBMN assay combined with kinetochore or centromere detection has been shown to be an optimal procedure for measuring whole chromosome loss events (*8–10*). Further technical developments (e.g., chromosome painting) may also allow the measurement of unequal distribution of chromosomes within daughter nuclei in cytokinesis-blocked binucleate cells. The scope for using the CBMN method in human population monitoring and recent advances in methodology are discussed below.

## Spontaneous Micronucleus Frequencies in Human Populations

Spontaneous or baseline MN frequencies in cultured human lymphocytes provide an index of accumulated genetic damage occurring during the lifespan of circulating lymphocytes. The half-life and mean lifespan of long-lived T-lymphocytes has been estimated to be 3 years and 4 years, respectively (*11,51*). The observed genetic instability may also reflect accumulated mutations in the stem cell lineage from which the mature lymphocytes originate.

The type of mutations that could contribute to spontaneous micronuclei include *a*) mutations to kinetochore proteins, centromeres, and spindle apparatus that could lead to unequal chromosome distribution or whole chromosome loss at anaphase, and *b*) unrepaired DNA strand breaks induced endogenously or as a result of environmental mutagens, which may result in acentric chromosome fragments. Studies using kinetochore antibodies to identify whole chromosomes suggest that approximately 50% of spontaneously occurring micronuclei are the consequence of whole chromosome loss and the rest are presumably derived from acentric chromosome fragments (*8–10*).

For the purpose of biological dosimetry, the spontaneous MN frequency refers to the incidence of MN observed in the absence of the environmental risk or exposure that is being assessed. The spontaneous MN frequency of a population has to be established to determine acceptable normal values as well as providing baseline data for those situations when spontaneous MN frequency for individuals is not known before exposure (e.g., in large-scale civilian accidents). The population database should *a*) enable an estimate of the induced MN frequency (i.e., observed minus expected spontaneous frequency) to be determined after an exposure accident, and *b*) given the establishment of dose–response parameters, an exposure dose can be calculated. The population database should span every decade up to 90 years and include a minimum of 20 individuals of each sex per decade. Ideally, this database should be updated regularly (every 2 years) to take into account the possible contribution of changing lifestyle factors as well as changes in technique. The database should also be used to identify lifestyle factors that can contribute significantly to the micronucleus index.

During an International Atomic Energy Agency-sponsored Research Coordination meeting for Radiation Biological Dosimetry held in Rio de Janeiro in June 1990, it was agreed that a compilation and comparison of baseline MN frequency data from various countries would be a useful exercise because it could enable a normal range of baseline values to be established as well as identify possible confounding factors. As part of our effort in this area, we have been collecting baseline data for the south Australian population. Our results on 225 individuals clearly suggest a significant positive correlation of MN frequency with age (Fig. 2). A comparison of the linear regression lines for males and females initially suggested that the slope and intercept for the relation of MN frequency with age was higher in females. To determine the significance of this observation and the possible effect of smoking, multivariate regression analysis was performed. The data set included information on 155 females and 70 males relating to age, sex, and smoking status including the number of cigarettes smoked per day and the number of years smoking. Only 29 of the individuals in the data set were smokers, and for 69 of the individuals, no information was available in relation to smoking. Initial analysis, ignoring the contribution of age and smoking, suggested that females do not have significantly higher MN frequency counts than males ($p > 0.20$). However, after adjusting for age, the difference between the MN frequency in males and females was highly significant ($p < 0.001$). After adjusting for age and sex, putting cigarettes in the model indicated that individuals with a high cigarettes/day ratio also have significantly elevated MN frequency counts when compared to low consumers ($p < 0.02$). However, the number of years smoking appeared to have no bearing on the observed MN frequency ($p > 0.20$). Other laboratories using the CBMN assay for population monitoring have also reported significant increments in MN frequency related to smoking (*12,13*) and aging (*12–15*). The reports by Yager et al. (*14*) and Migliore et al. (*15*) also show a very good agreement with the slope of the age-related increase in MN frequency reported in our studies (*4*). These data suggest that the CBMN methodology is reproducible in different laboratories and sensitive enough to detect the effects of lifestyle factors.

The study of spontaneous MN frequencies can also be performed on an individual basis. The spontaneous MN frequencies in individuals are particularly important in a

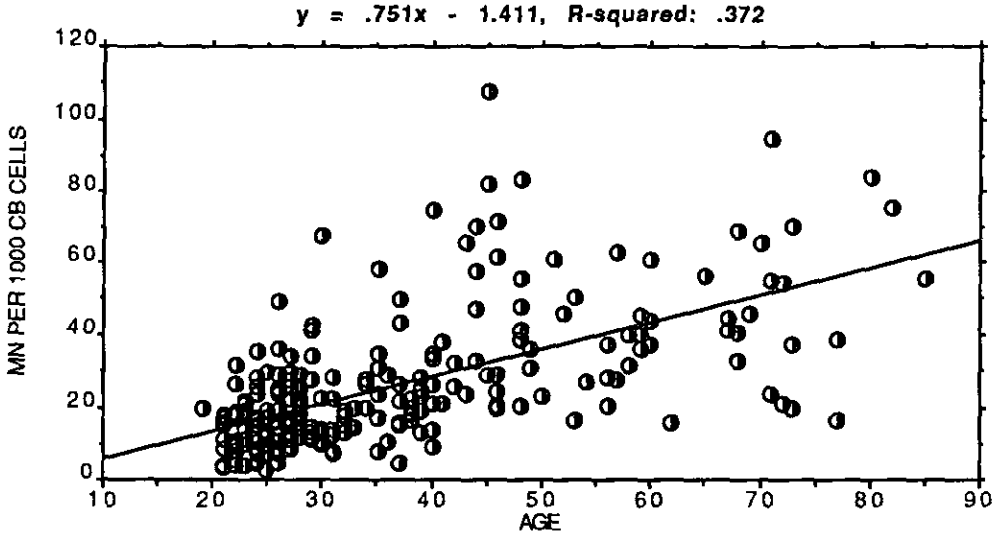


FIGURE 2. Spontaneous micronuclei frequency in cytokinesis-blocked lymphocytes of 224 healthy Australians plotted in relation to the age of the donor. The correlation between micronuclei frequency and age is statistically significant ($p < 0.0001$).

continuous monitoring program of individuals who are occupationally at risk of exposure to a powerful environmental mutagen such as radiation. In this case, individuals act as their own controls, and their MN frequencies are measured before and at regular intervals during their occupation. This situation is ideal because differences in lifestyle factors should not be a confounding factor. However, this approach is also dependent on the reproducibility of the assay; in other words, the assay should provide the same result when measurements are done on the same individual on separate occasions between which no significant exposure to mutagenic agents has occurred. Our own studies on baseline MN frequencies in cytokinesis-blocked binucleate lymphocytes of 12 normal individuals measured on two occasions 1 year apart show a good correlation between these measurements [$R = 0.845$, $p < 0.001$ (*16*)]. These type of results indicate that the CBMN methodology is a reliable and predictable method, but the residual level of uncertainty in MN frequency measurements should be taken into account when estimating risk. Furthermore, the relative incremental risk (RIR) after an exposure, estimated by the ratio of induced MN frequency and the spontaneous MN frequency, should also be measured because it accounts for the genetic damage that has already been accumulated in an individual before exposure due to age, lifestyle factors, or previous unknown exposure.

## Induction of Micronuclei in Cytokinesis-Blocked Cells after Exposure to Radiation or Chemicals

### Ionizing Radiation

Our own initial *in vitro* studies with the CBMN assay indicated quite clearly that if the baseline frequency of an individual was known before exposure, it was possible to detect increments in MN induction after exposures as low as 0.05 Gy (*4*). The detection limit could be further lowered if more binucleate cells were scored. In view of these encouraging data, we have evaluated the performance of this assay as an *in vivo* dosimeter in a recently completed study on micronucleus induction in cancer patients undergoing fractionated partial body radiotherapy at different sites with different irradiated volumes and integral doses (*17*). Measurements during radiotherapy have shown a dose-related increase in MN in all patients studied. The observed MN frequency correlated significantly ($p<0.0001$) with the equivalent body dose, which was estimated by dividing the integral dose by the body weight. This result suggests that the redistribution of lymphocytes had resulted in uniform exposure to the lymphocyte pool. The regression line for the relationship between equivalent body dose ($X$, in Gy) and the MN frequency ($Y$) is described by the equation:

$$Y = 75.8X + 49.5 \; (R = 0.783)$$

Further measurements were made periodically up to 12 months after treatment. Taken together, these results showed a general decline in MN frequency when compared to the frequencies at the end of treatment, decreasing to 91% after 3 months, 72% after 6 months and 57% after 12 months. These data are similar to those reported by Buckton et al. (*18*) for unstable aberrations in irradiated ankylosing spondylitis patients, thus supporting the theory that MN induced *in vivo* by ionizing radiation are mainly derived from acentric chromosome fragments.

Since our initial study, it has been shown that the CBMN assay can readily detect the *in vivo* clastogenic effects of combined radiation (accumulated doses of 10 cGy) and contrast media in patients undergoing excretory urography (*19*). Wells et al. (*20*) confirmed that the CBMN

assay can, indeed, readily detect exposures in patients undergoing fractionated partial-body radiotherapy, with the MN index being directly related to the integral dose. As a result of the possible application of the CBMN assay in biological dosimetry after radiation accidents, several laboratories around the world have now established the dose–response parameters after exposure to various sources of ionizing radiation (*3,4,6,7,20–25,29*). Furthermore, the Sobel's parallelogram concept of risk estimation (*26*) is also being implemented to determine the kinetics and extent of MN expression using the mouse as a model (*27,28*).

Our own data have shown that during the 14 days after whole-body irradiation, the MN frequency in spleen lymphocytes declined gradually to approximately half of the value observed immediately after irradiation. By contrast, the MN frequency in peripheral blood lymphocytes increased during the week after irradiation, but ultimately MN frequencies in the blood and spleen became approximately the same by day 14 and did not differ significantly from the MN frequency observed in blood lymphocytes immediately after exposure (*27*). Such data suggest that reliable dose estimates with the CBMN assay can still be obtained up to 2 weeks after whole body irradiation.

It has been suggested that the application of the CBMN assay in biological dosimetry after radiation accidents may be compromised by the relatively high background frequency (*29,30*). However, this criticism is diminished by the fact that *a*) it is comparatively easy to determine a database for baseline MN and define normal population levels of MN frequency, and *b*) exposure levels that are of clinical importance induce far more MN than what occurs spontaneously.

## Chemicals

The CBMN assay is increasingly being applied in population studies when significant *in vivo* exposures to genotoxic chemicals are suspected. Some of these studies have been particularly useful, as they have allowed direct comparison of the CBMN assay with other cytogenetic end points. These investigations included occupational exposure to styrene (*31,32*), chemicals in tannery industries (*33*), cyclophosphamide (*34*), and intervention trials with paracetamol (*35*). In three of these studies (*32,33,35*), increases in chromatid-type aberrations were reported; however, there was no corresponding increase in the MN index.

On the other hand, a recent report by Osanto et al. (*36*) has clearly shown that the CBMN assay can detect chromosome damage in the peripheral blood lymphocytes up to 9 years after chemotherapy of patients with testicular carcinoma. The overall impression that is gained from these studies is that the CBMN assay may be relatively insensitive to exposures to chemicals or agents that are either S-phase dependent or mainly induce covalent adducts on DNA.

## Relative Insensitivity of the CBMN Assay to Agents That Induce Covalent DNA Adducts but Not Strand Breakage

The cytogenetic lesions that are commonly increased in cells that have divided once after *in vivo* exposure to S-phase-dependent chemicals are chromatid-type aberrations such as chromatid deletions, achromatic lesions or gaps, and sister chromatid exchanges (*11,37*). The molecular mechanisms responsible for these lesions are not clearly understood; however, it is generally considered that a deletion is the consequence of a double-strand break event, whereas an achromatic lesion or gap may represent a base damage site or a single-strand break that does not result in a complete discontinuity in chromosome structure. As a consequence, of the various chemically induced cytogenetic aberrations, only chromatid or chromosome deletions can convert to lagging chromosome fragments at anaphase and be expressed as a micronucleus. The possible mechanisms underlying chemically induced DNA damage and the repair of covalent adducts suggest that there may be opportunities to improve the conversion of chemically induced base damage to micronuclei. An appropriate method would enable the conversion of adduct sites to single strand breaks in the $G_0$ or $G_1$ phase of the first division cycle. Single-strand breaks should convert to double strand breaks after the first S-phase and, consequently, to chromatid breaks and micronuclei by the time the first nuclear division is completed. During the past year, we have concentrated our efforts on developing an appropriate procedure for this approach.

## Conversion of Excision-Repairable DNA Lesions to Micronuclei Within One Cell Cycle

The opportunity for enhancing the sensitivity of the CBMN assay to genotoxins that mainly induce chemical adducts on DNA has arisen from studies over the last decade that have shown that cytosine arabinoside (ARA) can greatly enhance induction of chromosome aberrations in human lymphocytes after chemical exposure *in vitro* (*38–40*). ARA exerts its effect by inhibiting the DNA polymerization step (gap filling) that normally occurs after excision of damaged bases during nucleotide excision repair (long-patch repair) or base-excision repair (short-patch repair) (*41,42*). As a consequence, excision repair sites are converted to single-strand breaks. Preston and Gooch (*39*) suggested that it is probable that the interaction of these breaks, or their conversion to double-strand breaks after replicative DNA synthesis, could be the mechanism whereby synergistic increments in chromosome aberrations are produced by ARA after exposure to methylating agents, 4-nitroquinoline-1-oxide, or ionizing radiation. In principle this approach should also enable the conversion of excision repair sites to MN within one cell

cycle because acentric chromosome and chromatid fragments are readily converted to MN due to lagging at anaphase (Fig. 3).

The aim of our approach was therefore to combine the ARA methodology with the CBMN assay (*43*). The effect of ARA on MN induction was determined for human lymphocytes exposed *in vitro* to ionizing radiation and two agents that predominantly produced excision-repairable lesions but were weak inducers of MN in the standard CBMN assay, namely, methylnitrosourea (MNU) and ultravoilet light (UV, 254 nm). In the protocols used, the cells were incubated with ARA for most of the $G_1$ phase after exposure to the genotoxin to ensure that *a*) the maximum number of excision sites were converted to single-strand breaks, and *b*) to minimize the possibility of gap-filling after removal of ARA before S-phase started (Table 1). The induced MN frequencies (i.e., observed MN frequency in mutagen-treated culture minus MN frequency in corresponding control) in cultures without ARA and equivalent cultures treated with ARA were compared. The results (Figs. 4–6) showed that ARA treatment increased the slope of the dose–response curve for induced MN frequency for X-rays, UV, and MNU by a factor of 1.8, 10.3, and 40.0, respectively. Furthermore, a 24-hr gap between mutagen exposure and the start of the assay did not abolish the increased mutagen sensitivity in the cultures that were also treated with ARA, suggesting that the method would be of value in *in vivo* monitoring even when blood samples cannot be obtained immediately after exposure. Exposure to ARA alone also provides a tool for monitoring endogenously occurring events such as excision repair of base hydroxylation due to free-radical damage. Our initial baseline data also suggest that there is a negative correlation ($R = 0.584$, $p = 0.002$) between the age of donor and the ARA-induced MN frequency in control cultures probably due to a decline in excision repair with age (*43*). This preliminary work clearly suggests that the combined ARA–CBMN assay can considerably improve the role of the micronucleus assay in biological dosimetry and screening of new chemicals introduced into the environment. However, due to its inherent sensitivity and until we can better understand the possible confounding factors influencing interindividual variability in ARA-induced MN, it would seem prudent, at this early stage, to limit the application of the combined ARA–CBMN assay only to those studies where individuals can act as their own controls.

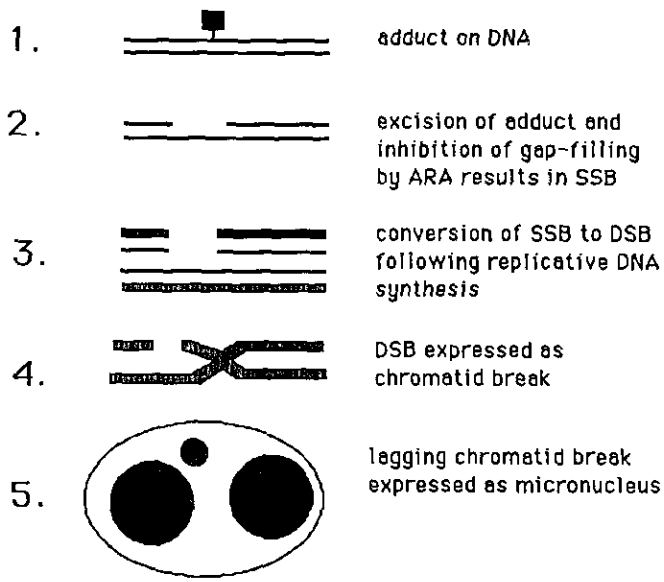


FIGURE 3. A schematic diagram explaining a possible mechanism for the conversion by cytosine arabinoside of an excision-repairable DNA lesion to a micronucleus within one cell cycle. SSB, single-strand break; DSB, double-strand break; ARA, cytosine arabinoside.

**Table I. Protocol for the conversion of excision-repairable DNA lesions to micronuclei within one cell cycle in human lymphocytes.**

| 0 hr | 16 hr | 44 hr | 72 hr |
|---|---|---|---|
| 1. Stimulate with PHA | 3. Wash 2X with HBSS | 5. Add CYT-B | 6. Harvest cells |
| 2. Add ARA (control without ARA) | 4. Transfer to fresh medium + CM + DC | | |

Abbreviations: PHA, phytohemagglutinin; ARA, cytosine arabinoside at 1 μg/μL; HBSS, Hanks' balanced salt solution; CM, conditioned medium as a source of interleukin-2; DC, deoxycytidine at 10 μg/mL; CYT-B, cytochalasin-B.

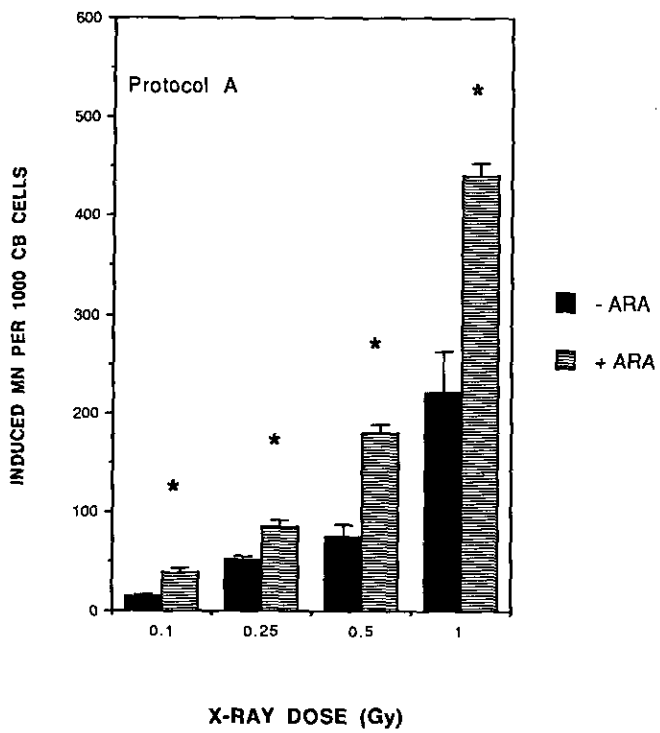


FIGURE 4. X-ray induction of micronuclei in cultures without cytosine arabinoside (ARA) (−ARA) and cultures with ARA (+ARA). The vertical lines represent the SE of the mean (* $p < 0.02$, $n = 4$).

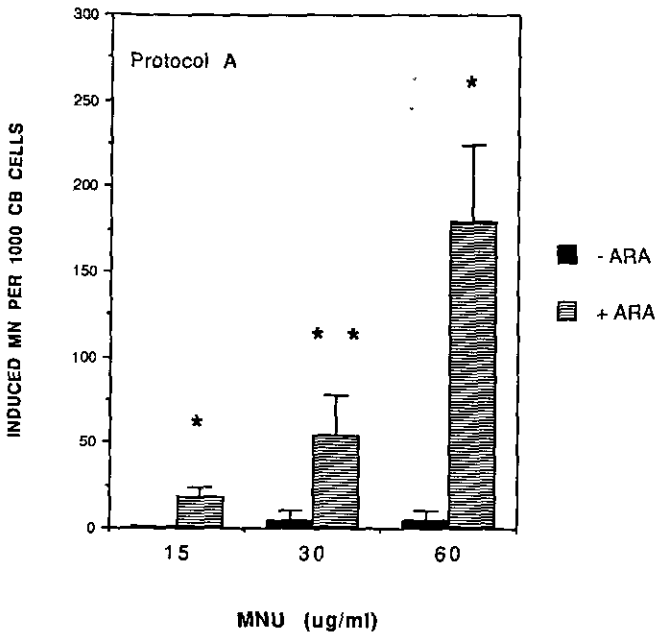


FIGURE 5. Methyl nitrosourea induction of micronuclei in cultures without ARA (− ARA) and cultures with ARA (+ ARA). The vertical line represents the SE of the mean (* $p < 0.03$, ** $p < 0.10$, $n = 4$).

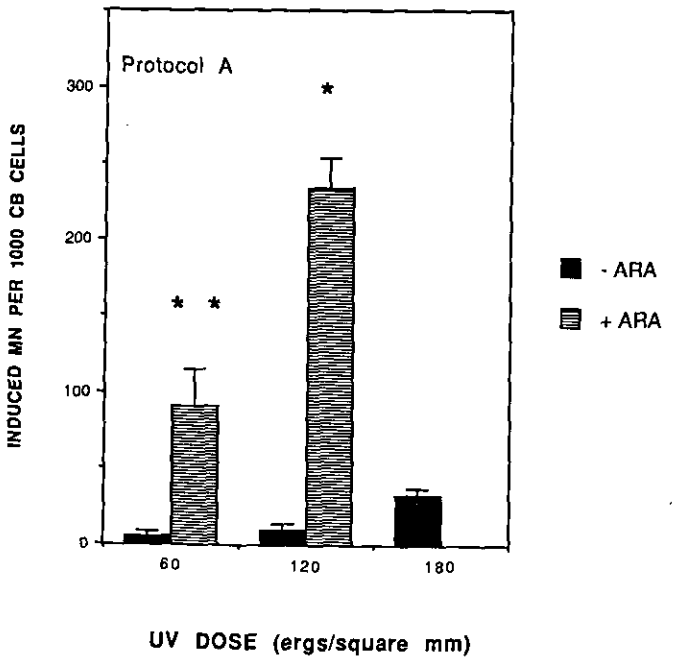


FIGURE 6. UV induction of micronuclei in cultures without cytosine arabinoside (−ARA) and cultures with ARA (+ARA). It was not possible to determine the micronuclei frequency in + ARA cultures at the highest UV dose because this combination made all the cells necrotic. The vertical lines represent the SE of the mean (* $p < 0.01$, ** $p < 0.06$, $n = 3$).

## Conclusion

Since the initial description of the CBMN assay, there have been several reports that have enabled a better definition of its potential for biological dosimetry. The general consensus seems that the CBMN assay, due to its relative ease of implementation and its ability to complement other cytogenetic assays, has an important role to play in biomonitoring genotoxic exposure. However, there also appears to be some concern that the baseline MN frequency in human populations requires better definition if this technique is to effectively discriminate between DNA damage occurring as a result of normal metabolic processes and genotoxic effects due to man-made or natural environmental agents. Our most recent investigations indicate that it is possible to markedly increase the sensitivity of the CBMN assay to genotoxins that mainly induce excision-repairable lesions (*43*), and Norman et al. (*44*) have recently demonstrated that the cytokinesis-block methodology can also be implemented to readily quantify HPRT variants, thus enhancing the role of this technique in mutagenesis research. Finally, one can look forward to further development of automation by image analysis (*45*), which should enhance the application of the micronucleus assay in routine screening programs such as assessment of interindividual variation in radiosensitivity (*46*), prediction of radiocurability of tumors (*47–50*), and generally in those studies of a molecular epidemiological design (*2*) that would require several hundred blood samples to be cultured for micronucleus measurement.

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